

MICROBIC DISSOCIATION OF
B. COLI COMMUNIS

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ANNA DEAN DULANEY, A. B., A. M.

*The Abstract of a Thesis Submitted in Partial Fulfilment
of the Requirements for the Degree of
Doctor of Philosophy*

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MICROBIC DISSOCIATION OF *B. COLI COMMUNIS**

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The term microbic dissociation has recently become significant because it logically connects some of the various expressions of bacterial instability as developmental phases of one phenomenon,—namely bacterial cyclogeny. It is now used to designate certain more or less ordered modifications in bacterial cultures,—changes which in many cases, may be predicted and controlled, and which are intimately associated with cell morphology, colony form, virulence, biochemical behavior and serologic reactions.

Microbic dissociation can probably be demonstrated in all species of bacteria. It would seem that, underlying the dissociation reaction are fundamental governing forces which relate to the reproductive mechanism and are common to all bacterial protoplasm; moreover, that an understanding of this mechanism, and an interpretation of its significance, could best be derived from a study of the process of dissociation in related and unrelated organisms. The following contribution represents a study of dissociation in *B. coli-communis*. The cultures used were laboratory strains from human sources which had been isolated within a period of two years previous to the time of study, and which gave typical fermentational and other cultural reactions.

In view of Hadley's recent work¹ a review of the general literature seems unnecessary. He has correlated the contributions preceding and following those of Arkwright² and de Kruif,³ whose work first stimulated interest in the subject of microbic dissociation as a distinct phenomenon (although without indicating its widespread significance or giving to the phenomenon a satisfactory interpretation) and in addition has offered a theory suggesting a relationship of the phenomenon to bacteriophagy—the so called "homogamic theory." Since his publication Petroff⁴ has described the S and R forms of several strains of *B. tuberculosis*, Nungester⁵ has reported on certain dissociants of *B. anthracis*, and Tunncliffe⁶ has indicated the existence of dissociational variation in her streptococcus strains from measles, as have Bigger, Boland, and O'Meara⁷ for variants of *Staphylococcus aureus*.

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1. *J. Infect. Dis.*, 1927, 40, p. 1.
2. *J. Path. & Bact.*, 1921, 24, p. 36.
3. *J. Exper. Med.*, 1927, 33, p. 773.
4. *Proc. Soc. Exper. Biol. & Med.*, 1927, 24, p. 632.
5. *Proc. Soc. Exper. Biol. & Med.*, 1927, 24, p. 959.
6. *J. Infect. Dis.*, 1927, 41, p. 267.
7. *J. Path. & Bact.*, 1927, 30, p. 261.

CHIEF FORMS OF *B. COLI*

When a pure culture of this organism is grown in nutrient broth, P_H 7.0 for at least ten days at 37 C. and then plated, two distinct colony forms may be recognized. The S form is the one familiar to all bacteriologists, giving the round, regular-edged, dome-shaped opalescent colony. The growth on agar is smooth and moist, fluorescent, easily picked up with the inoculation needle. It readily forms a homogeneous suspension in 0.85 per cent solution of sodium chloride. The organisms are the usual short, slender rods, showing a slight but definite motility. In broth there is uniform clouding with a slight film around the edge.

The R form shows the characteristic features described for the R forms of other intestinal organisms by Arkwright² and later workers. The R colony is irregular, flat, dry, rough or granular, and dull by reflected light. The growth is bluish or translucent by transmitted light and there is no fluorescence. The growth on agar slants is dry and tends to cling to the inoculation needle. When it is suspended in 0.85 per cent NaCl there occurs spontaneous clumping. Even salt solutions as dilute as 0.21% fail to produce a stable suspension, although Arkwright² reported otherwise for Shiga cultures.

The R organisms are uniformly shorter and broader than the S and may be even coccoid. No motility has been demonstrated. In broth the growth is granular or "agglutinative" and the organisms tend to settle out, leaving the broth almost clear. The primary R colonies often show secondary or daughter colonies. These are easily seen with a hand lens and are found in colonies which are at least ten days old. These secondary colonies develop at or near the margin of the R colony and give a papillose appearance to the primary growth. Smears and cultures from these secondary colonies yield S forms.

Another type of colony often found on agar plates has been termed the RS colony. During the first 48 hours of incubation, this colony is apparently a true S form—round, regular, dome-shaped, and with a sheen. However, smears from such a colony will show a mixture of R and S forms with a predominance of R organisms, and cultures will confirm the smears. Later, after 48 to 72 hours—a distinct change in the edge of the colony may be observed. A thin, bluish, slightly irregular fringe or marginal zone, extending around the entire colony will develop; or in some cases only a portion of the periphery may become changed. In the latter case, either isolated wedge-shaped

"sectors" may appear, or irregular, connected areas may extend around perhaps a fourth of the circumference. Gratia⁸ noted these changes in *B. coli* colonies and Hadley¹ has termed this "marginal dissociation." Smears or cultures from these thin marginal or irregular areas will give R forms, while those from the central area will give either S forms, or a mixture of R and S forms. This colony will never take on the flat, dull, rough appearance; it will preserve its smooth appearance, but the growth will become opaque and the sheen decrease.

Although the S and R forms of organisms are the chief ones considered in studies on dissociation, an intermediate or transitional form has been described by various workers for certain species. This intermediate form which has been related to the O type culture was obtained by growing normal S cultures on 0.1% phenol agar for several generations according to the method of Braun and Schaeffer⁹ for *B. proteus*. The O variant was never stable, but readily returned to the S, from which it differed in antigenic structure and serological response. In other species, the O type has been reported as transforming to the R.

Hadley¹ believes that there is probably always an intermediate form between the S and R stages,—an "unstable culture form—apparently both the 'progeny' of the S and the 'progenitor' of the R." He suggests that "the R type is not a direct, but an indirect product of S." The O form is, however, difficult to detect in many species.

I have demonstrated a highly unstable form of *B. coli* by means of cultivation of the S form on 0.1% phenol agar and by analogy this may be regarded as the O form. The O colony in general appearance suggests the S. It is larger, however, somewhat mucoid, and lacks the sheen, although it cannot be termed dull. A striking change in the morphology of the organisms takes place after growth on phenol mediums. The bacilli appear swollen, irregular in size and shape; some are very short, while others are long and distorted. The maximum change occurs after two or three passages on phenol medium. The growth on plain agar is smooth and luxuriant and is readily suspended in 0.85% NaCl. In broth there is homogeneous cloudiness. This O form is highly unstable and no permanent O cultures have been obtained. Early forms are more S-like while later forms resemble the typical R organisms, thus suggesting its intermediate nature. The O form as a recognized stage in the cyclogeny of *B. coli* demands further study.

8. J. Exper. Med., 1922, 35, p. 287.

9. Ztschr. f. Hyg., 1919, 89, p. 339.

FACTORS RESPONSIBLE FOR DISSOCIATION

Aging.—The first recognized and commonly used means of securing what we now term dissociants,—that is, by colony selection from plates streaked from old broth, gelatin, or agar cultures of the “normal” form—affords a sure and satisfactory means of obtaining R forms of *B. coli-communis*. Broth cultures of the S form were allowed to stand at room temperature for several weeks or months, then streaked on alkaline (P_H 8.0) agar plates. The percentage of R colonies obtained is in direct relation to the age of the culture. Broth cultures which had stood for eight weeks gave a high percentage of R colonies while 100% of R forms were obtained after ten weeks.

While the reaction of the culture medium will be considered later it may be stated here that plain broth cultures of both S and O of initial reactions ranging from P_H 5.2-8 will show R forms after a proper period of aging. As Hadley¹ suggests, the R form seems to be the “ultimate goal” in the transitional process.

Hydrogen-Ion Concentration of Culture Medium.—The final hydrogen-ion concentration of the culture medium definitely influences the dissociative process. In *B. coli-communis* an alkaline reaction promotes, while an acid medium completely inhibits dissociation into the typical R state. When this culture is grown in plain broth the final reaction is alkaline. Dissociation under these conditions consistently takes place. When, however, fermentable sugar is added to the culture medium dissociation fails to occur, due presumably to the acid which is produced by the utilization of the sugar. A striking illustration of these facts is demonstrated by the following experiment.

Exper. 1:—Three tubes (each containing 5 cc.) of each of four broths—plain unbuffered, plain buffered, lactose buffered and lactose unbuffered—were adjusted to P_H 5.2, 7.1 and 8.4 respectively.

Phosphate buffer mixtures prepared according to Clark¹⁰ were used. Plain broth refers to standard beef extract broth. Lactose broth refers to plain broth to which 1% lactose was added.

All of the tubes were inoculated with the S culture, and transplants made at 48-hour intervals for 10 days. The last set of transplants after an incubation of 24 hours at 37.5 C. was left at room temperature, and alkaline agar plates were streaked from these cultures at intervals. Table 1 shows that the plain broth—both buffered and unbuffered—and of all reactions, soon contained R forms, while the lactose broth continued to yield only S forms.

It was further found that the organisms died out much sooner in the lactose broth. After 76 days the plain broth tubes still yielded good growth upon transplantation, while no living organisms could be demonstrated in any of the tubes of

10. The Determination of Hydrogen-Ions, 1922.

lactose broth. It would seem that the addition of lactose encouraged more rapid and luxuriant growth, with acid formation, and that the organisms were killed by the products of their own metabolism. The organisms in the plain broth grew more slowly, the products of metabolism were thus accumulated less rapidly, and the life of the culture materially prolonged.*

Temperature and Moisture.—Temperatures from 42 to 45 C. definitely stimulated dissociation of *B. coli*. This observation is in accord with the findings of older workers, and with the recent reports of Soule¹¹ on *B. subtilis*, and of Tunnicliff⁶ on diplococcus strains from measles. The use of very dry solid culture mediums also encouraged the S→R change.

Immune and Normal Serums.—In addition to these methods of producing dissociation it was found that cultivation in plain broth to

TABLE 1.—THE RELATIONSHIP BETWEEN DISSOCIATION AND THE FINAL PH OF THE CULTURE MEDIUM

Broth Mediums	Original P _H	Colony Form Obtained on Plates			P _H after 60 Days
		10 Days	30 Days	50 Days	
Plain unbuffered.....	5.2	No growth	No growth	No growth	5.2
	7.1	S only	Many R, few S	100% R	7.8-8
	8.04	S only	Many R, few S	95% R	8.8
Plain buffered.....	5.2	S only	Many R, few S	100% R	6.2-6.4
	7.1	S only	Many R, few S	100% R	7.8-8
	8.04	S only	Many R, few S	90% R	8.8
Lactose unbuffered.....	5.2	S only	S only	S only	4.4
	7.1	S only	S only	S only	4.4
	8.04	S only	S only	S only	4.4-5
Lactose buffered.....	5.2	S only	S only	S only	4-4.4
	7.1	S only	S only	S only	4-4.4
	8.04	S only	S only	S only	4.4-5

which 10% of homologous immune serum had been added readily produced dissociation of the S form. This interesting fact which was first pointed out and related to the dissociative reaction by Griffith,¹² in his studies on the pneumococcus, has been confirmed by the later work of Reimann,¹³ Julianelle,¹⁴ Soule,¹¹ and others.

Neither heterologous nor normal serum in any concentration produced the S→R change in *B. coli*. Fresh normal serum with complement undestroyed was no more effective than was normal serum which had been heated for 1 hour at 55 C.

* These results raise the interesting question regarding the cause of death of bacteria in acid culture mediums. Is it because they cannot dissociate into the R cyclostage?

11. *J. Bact.*, 1927, 8, p. 41.

12. Report 18 on Public Health and Medical Subjects, Ministry of Health, 1923.

13. *J. Exper. Med.*, 1925, 41, p. 587.

14. *Ibid.*, 1926, 44, p. 683.

It was interesting to find that the immune serum developed by using as antigen an S culture which had been heated for two hours at 100 C. was somewhat more effective in stimulating the S $\rightarrow$ R change than was the immune serum prepared by using as antigen the S culture heated to 55 C. for 1 hour. While relative differences in the strengths of the two serums will probably explain this result, it is significant since it seems to suggest that the antigenic component of *B. coli* responsible for type specificity of immune serum is heat stable.

When 1% lactose broth was used instead of the plain broth the S immune serum was ineffective. As shown in the preceding experiment, an acid medium prevents dissociation of the S form, and the specific

TABLE 2.—THE PERCENTAGE OF COLONIES OF DIFFERENT TYPES ON ALKALINE AGAR FROM S CULTURES GROWN IN VARIOUS SERUM-BROTH MEDIUMS

Time in Days	S Serum	R Serum	Heated Serum*	Heated Normal Serum	Fresh Normal Serum	Lactose Broth - S Serum
1	98 S 2 R	100 S	100 S	100 S	100 S	100 S
2	95 S 5 R					
5	5 S 95 R	100 S	5 S 95 R	100 S	100 S	100 S
8	100 R		100 R			
10	100 RS	100 S	60 S 40 R	100 S	100 S	100 S
12	100 RS	100 S		100 S	100 S	100 S
15	100 RS	100 S	70 S 30 R	100 S	100 S	100 S
20	100 RS	100 S	90 S 10 R	100 S	100 S	100 S

* S serum heated at 100 C. for 2 hours.

action of the immune serum was not effective under such conditions. The influence of serums on the stage in cyclogeny is demonstrated in the following experiments.

Exper. 2:—S and R immune serums, heated normal serum, fresh normal serum, and S immune serum prepared by using as antigen an S suspension heated for 2 hours at 100 C., were added to plain broth in a series of tubes of plain broth in the proportion of 1:10. S immune serum was also added to tubes of 1% lactose broth in the proportion of 1:10. The total volume of medium in each case was 2 cc. All tubes were then inoculated with S culture, incubated at 37.5 C. and serial transplants made daily for 20 days. Alkaline agar plates were streaked from those cultures at intervals.

Table 2 shows, first, that the S serum readily brought about the dissociation of the S culture to the R state, but that the product of transformation was not permanent. After 10 days the plates gave the RS type of colony, which may be considered an intermediate stage,

and continued to show this form. Griffith¹² has emphasized the importance of the titer of the serum on the permanence of the R form, and even though this $S \rightarrow R \rightarrow S$ change was obtained by use of several different S serums, it may be found that serums of higher titers will produce a more stable R type.

Table 2 also shows that a 10% concentration of normal rabbit serum was ineffective in promoting dissociation of the S culture. If dissociation actually takes place in vivo, as sometimes would seem to be the case, it might be expected that a greater concentration of normal serum would stimulate this dissociation, especially if daily transfer provided fresh contact with the serum. When, however, experiments on this point were conducted, pure serum was found no more effective than the 10% dilution, as shown below.

Exper. 3:—Normal rabbit serum which had been heated for 1 hour at 55 C. was added to beef extract broth in amounts ranging from 10 to 90%. Undiluted serum was also used. The total volume of medium in each case was 5 cc. All of the tubes were inoculated with the S form of *B. coli*, the cultures left at 37.5 C. for 14 days and frequent platings made on alkaline agar plates.

The plate cultures—made 1 day, 3 days, 6 days and 14 days after inoculations of the rabbit serum-broth solutions—all had 100% S colonies which shows that normal rabbit serum in any concentration did not stimulate dissociation of the S type culture in vitro within the time limit and volume employed.

FACTORS PRODUCING THE $R \rightarrow S$ CHANGE

The $S \rightarrow R$ change can probably be demonstrated in all bacterial species. If the $S \rightarrow R$ change is permanent, the R form might be accepted as an actual mutant. If the $S \rightarrow R$ change does not involve mutation, the $R \rightarrow S$ phenomenon should be capable of demonstration by suitable methods. Conflicting evidence has been offered by various workers.

De Kruif regarded his G form of *B. leptosepticum* as a mutant, and this has been a common interpretation in such cases. Schutze¹⁵ found it impossible to produce a reversion of the R form of *B. paratyphosus* B., and Amoss¹⁶ accepted the permanence of the R form of pneumococcus. On the other hand, Jordan¹⁷ has reported a reversion of the R form of *B. paratyphosus* B., as has Soule¹¹ for *B. subtilis*.

15. Brit. J. Hyg., 1922, 20, p. 330.

16. J. Exper. Med., 1925, 41, p. 649.

17. J. Am. M. A., 1926, 86, p. 177.

It was found that the "early" R forms of *B. coli communis* readily changed to the S. Whether this change represents a reversion or a progression (as Hadley¹ suggests—), recently dissociated R forms soon acquired the characteristics of the typical S under any condition of optimum environment. This suggests that the "early" R may have been an O form with R characteristics. When the R form had become stabilized, however, the $R \rightarrow S$ transformation was brought about only by cultivation in lactose broth, which provided ultimately a distinctly acid medium, or in broth containing homologous R immune serum.

Frequent Transplantation.—Jordan¹⁷ found that frequent transplantation in veal infusion broth, P_H 7.4, produced the reversion of an R strain of *B. paratyphosus* B. The culture was transferred twice each day and after 25 days a large percentage of S colonies was obtained on plates. This method did not produce the $R \rightarrow S$ change in *B. coli* but instead, seemed to result in greater stabilization of the R cyclostage, as shown by the following experiment.

Exper. 4:—An R culture which had consistently yielded 100% R colonies for many generations was transplanted to tubes containing 5 cc. of beef infusion broth, P_H 7.0, twice each day at approximately 12-hour intervals. The cultures were incubated at 37.5 C. Alkaline, plain agar plates were streaked each evening at the time of transfer. After 40 days, the plates still showed 100% R colonies.

Large Volumes of Broth.—Feiler¹⁸ found that the $R \rightarrow S$ change in *B. typhosus* took place more rapidly in large volumes of broth (50 cc. amounts). Soule¹¹ confirmed these results and observed that while both the $S \rightarrow R$ and the $R \rightarrow S$ transformations took place more rapidly in large volumes of broth (300 cc. amounts), dissociation of S occurred at a faster rate than did reversion. This point was studied as follows.

Exper. 5:—Two flasks each containing 200 cc. of beef infusion broth, P_H 7.0, were inoculated with the R and S forms respectively of *B. coli*. After 24 hours at 37.5 C alkaline agar plates were streaked and another set of flasks inoculated. This was repeated six times. All plates gave 100% R colonies even after 6 days.

Normal Serum.—De Kruif³ found that the G (=R) form of *B. leipsecicum* persisted after prolonged cultivation in 5% normal serum broth as well as in plain broth. Recently, however, Soule¹¹ has obtained a rapid reversion of the R type of *B. subtilis* by cultivation in large volumes of beef infusion broth to which was added 5% active normal serum.

18. Ztschr. f. Immunitätsforsch. u. exper. Therap., 1920, 29, p. 303.

Since the addition of small amounts of normal serum to culture mediums is conducive to the maintenance of the S characteristics, the $R \rightarrow S$ change might be expected to take place under such conditions. This was not demonstrated with *B. coli* as shown by the following test.

Exper. 6.—Normal rabbit serum which had been heated for 1 hour at 55 C. was added to beef infusion broth, P_H 7.0, in the proportion of 1:20. The R culture was transplanted to tubes containing 5 cc. of this serum broth twice each day, and the cultures incubated at 37.5 C. Alkaline agar plates were streaked each day at the time of transfer. After 15 days the plates still showed 100% R colonies.

Lactose Broth.—It has been shown in preceding experiments that an acid reaction produced by the utilization of lactose prevented the dissociation of the S form of *B. coli*, even when homologous immune serum was added to the culture medium. Might an acid medium then be expected to bring about the $R \rightarrow S$ transformation? This fact was demonstrated by rapid passage of the R culture in 1% lactose broth, as follows.

Exper. 7:—The R culture used in the above experiments was transplanted twice each day through a series of tubes containing 5 cc. of beef infusion broth, P_H 7.0, to which had been added 1% of lactose. The cultures were then incubated at 37.5 C. Alkaline agar plates were streaked each evening at the time of transfer. The following table shows that the R forms were soon replaced by the S, as indicated by the relative proportion of S and R colonies.

TABLE 3.—THE PERCENTAGE OF S AND R COLONIES AFTER CULTIVATION OF THE R FORM IN LACTOSE BROTH

Type of Colony	Days	Relative Number of Colonies after Number of Days Indicated								
		2	3	4	5	6	7	8	9	10
S.....	0		1	2	3	5	15	30	40	50
R.....	All R		3	1	1	1	1	1	1	1

The P_H of the broth changed from 7.0 to 4.6 in the 12 hour incubation period, thus providing an acid reaction which is evidently as favorable to the $R \rightarrow S$ change as to the maintenance of the S form. It was further found that when the R culture which had been transferred twice each day for 40 days in beef infusion broth (exper. 4) and which continued to give 100% R forms was subjected to rapid passage in lactose broth no S colonies were found until the tenth day. With greater stabilization of the R form the $R \rightarrow S$ change was brought about much more slowly even in acid medium.

Homologous Immune Serum.—Although the specific effect of homologous S serum on the dissociative reactions of the S culture had been observed by various workers, a study of the effect of R serum

on the reversion of the R form was not made until very recently. This was done by Soule¹¹ who found that a rapid reversion of an R strain of *B. subtilis* took place when this organism was cultivated in broth to which 10% R immune serum had been added.

This interesting fact was confirmed by the response of the R form of *B. coli* studied in this laboratory to the specific action of the R immune serum. While the reversion of *B. coli* was not nearly so rapid as that obtained by Soule¹¹ (40% in 24 hours) a distinct change in colony form was demonstrated upon cultivation in contact with homologous immune serum, as shown below.

Exper. 8:—The R culture was daily transplanted to broth to which 10% R immune serum had been added, and to broth to which S immune serum had been added in the same proportion. The following table shows that homologous immune serum produced the R → S change, but that the heterologous immune serum had no such effect. It was found that 10% concentrations were as effective as those of 25 and 50%.

TABLE 4.—THE PERCENTAGE OF COLONIES OF DIFFERENT TYPES ON ALKALINE AGAR PLATES FROM R CULTURES GROWN IN VARIOUS SERUM-BROTH MEDIUMS

Serums Used	Days:	Percentage of Colony Types Present after					
		1	2	3	5	10	15
R serum.....		100 R	90 R	80 R	75 R	50 R	10 R
		0 S	10 S	20 S	25 S	50 S	90 S
S serum.....		100 R	100 R	100 R	100 R	100 R	100 R

VIRULENCE

A marked difference in the virulence of the S, O, and R forms of *B. coli* communis was demonstrated. Hadley¹ has pointed out that this is the general rule in many species.

The lethal dose of R culture was found to be 15 times that of S culture. When the R culture produced death in guinea-pigs, the R organisms were recovered from the peritoneal exudate. This confirms de Kruif's³ report that comparatively large amounts of his G (=R) cultures were fatal to young rabbits and that the G form was isolated at autopsy.

It was also found that the O forms which had been produced by growth on phenol agar for two generations and which were still distinctly S-like retained their virulence to a large extent while the O forms which had been carried on phenol agar for ten generations, and which closely resembled the R type, showed the avirulent properties of this form.

When living cultures were used for immunization of rabbits, the S organisms in every case produced abscesses upon subcutaneous

injection while the R organisms caused no such results. These abscesses broke down and healed promptly and apparently did not injure the rabbits, but they indicated a distinct difference in the toxic properties of the two forms.

Immunization with the R form protected guinea-pigs from lethal doses of S. De Kruif³ found that rabbits inoculated with G (= R) cultures of *B. leprosepticum* resisted lethal doses of D (= S) and Mary Cowan¹⁹ has emphasized the protective properties which immunization with R forms of streptococci confer on mice. On the other hand, Arkwright²⁰ has concluded that the immunization of rabbits with the R form of *B. paratyphosus* B. gives slight protection. Since injection of the R cultures produces no such toxic effects as those produced by the

TABLE 5.—DIFFERENCES IN VIRULENCE OF S, O, AND R FORMS FOR GUINEA-PIGS

Culture	Dose, Cc.	Results	Necropsy Findings
S.....	1.0	Death, 15 hours	Purulent peritonitis S culture isolated
S.....	0.8	Death, 18 hours	Purulent peritonitis, S
S.....	0.5	Death, 22 hours	Purulent peritonitis, S
S.....	0.5	Death, 22 hours	Purulent peritonitis, S
S.....	0.1	Death, 30 hours	Purulent peritonitis, S
O ¹	1.0	Death, 24 hours	Purulent peritonitis, S
O ²	1.0	Survived	
R.....	1.0	Survived	
R.....	1.5	Survived	
R.....	1.8	Survived	
R.....	2.0	Survived	
R.....	2.5	Death, 23 hours	Purulent peritonitis R culture isolated

¹ Early O form, - S like.

² Late O form, - R like.

S, and at the same time stimulates a definite immunity, the practical use of R cultures for bacterial vaccines might be suggested in some cases. The familiar "reactions" may be found to be due to the toxic S properties which may thus be eliminated. On the contrary, certain recent work (Felix and Olitzki),²¹ (Arkwright)²⁰ seems to suggest a greater protective value in the O antigen. In the present experiments this antigen was not tested for protection.

Exper. 9:—A series of guinea-pigs, averaging 500 grams, was used for the virulence tests. Saline suspensions of 15 to 24 hour agar cultures, diluted to a uniform turbidity were injected intraperitoneally. Table 6 shows a distinct difference in virulent properties.

Exper. 10:—A series of guinea-pigs, averaging 500 grams, was given intraperitoneal injections of living R cultures of *B. coli* at 4 day intervals. Five days after the last injection of R, the guinea-pigs received a more than lethal dose of living S

19. Brit. J. Exper. Path., 1924, 5, p. 226.

20. J. Path. & Bact., 1927, 30, p. 345.

21. J. Immunol., 1926, 11, p. 31.

culture also given intraperitoneally. The following table shows that a definite protection was afforded by immunization with R.

CULTURAL REACTIONS

In most respects the S and R forms of *B. coli-communis* gave identical cultural reactions. The results with sugars—dextrose, lactose, maltose, mannitol, sucrose—and with inulin, dulcitol, milk, gelatin, were identical. Nitrate reduction, ammonia and indol formation were the same for both forms. A distinct difference, however, was found in the reactions which the stabilized R form gave on Endo plates and in Russell's double sugar tubes. While the S organism produced the typical acid reaction with characteristic metallic sheen on Endo plates the R colonies remained colorless. On Russell's double sugar medium the S form soon gave the typical *B. coli* picture while with the R organism the slant either remained unchanged or developed very slowly a faint acid reaction. In view of the routine

TABLE 6.—THE PROTECTIVE VALUE OF IMMUNIZATION WITH R AGAINST LETHAL DOSES OF S

Guinea-Pig	Immunization, R	Infection, S	Result
1	1 injection, 1 cc.	0.5 cc.	Died
2	2 injections, 1 cc.	1 cc.	Survived
3	3 injections, 1 cc.	1 cc.	Survived
4	4 injections, 1 cc.	1.5 cc.	Survived

use of Endo agar in water examinations this result assumes distinct significance.

IMMUNE REACTIONS

Modifications in serologic behavior have constituted an important phase of all dissociation studies. Arkwright² reported distinctly different antigenic properties for his S and R forms, while de Kruif's³ findings indicated only quantitative differences in immune responses. Later papers by Arkwright and Goyle²² (intestinal bacteria), Goyle²³ (*B. typhosus* and *B. enteritidis*, Gärtner), Balteanu²⁴ (cholera), Reimann¹³ (pneumococcus), Julianelle¹⁴ (Friedländer's *Bacillus*) and others, offer proof of a characteristic alteration in antigenic components with the S → R change.

Detailed results of serological studies of *B. coli* will be presented in a later paper, but it may be stated here that the S and R forms showed distinctly different antigenic properties. The S culture when injected into rabbits stimulated immune responses for both S and R, while the

22. Brit. J. Exper. Path., 1924, 5, p. 104.

23. J. Path. & Bact., 1926, 29, p. 149.

24. Ibid., 1926, 29, p. 251.

R form produced antibodies for the R culture only. The R organism had lost a property possessed by the S.

The S immune serum agglutinated both S and R forms, but agglutinated R in lower dilutions than S. The R immune serum contained no agglutinins for S, and agglutinated R at a relatively low titer.

A characteristic difference in the types of agglutination was given by the two forms. The unheated S culture was clumped in a flocculent manner while a finely granular, irregularly spreading deposit was formed by the unheated R antigens. A study of the agglutination of heated antigens is in progress.

In absorption tests the S antigen removed all agglutinins for both S and R from the S immune serum, while only the R agglutinins were removed from S serum after two absorptions with R antigen. However, the S agglutinins were reduced by such treatment. When S antigen

TABLE 7.—RESULTS OF AGGLUTINATION TESTS WITH S AND R SERUMS BEFORE AND AFTER ABSORPTION

Immune Serum	Results	
	S	R
S unabsorbed.....	1280	640
S unabsorbed *.....	1280	640
R unabsorbed.....	0	640
S absorbed with S.....	0	0
R absorbed with S.....	0	0
S absorbed with R.....	980	0
R absorbed with R.....	0	0

* Immune serum developed with heated antigen.

was heat stable. When 24 hour cultures of *B. coli-communis* were removed, and the R antigen after two absorptions likewise removed the R agglutinins. The results of these tests are shown in table 7.

The effect of heating on the antigenic properties of S was studied. S antigen was heated in an Arnold sterilizer for 2 hours at 100 C. and then injected into rabbits. The immune serum thus produced gave the same reactions with S and R unheated antigens as did the serum produced by unheated S antigen. These results with such immune serum represent preliminary studies, but they seem entirely reasonable since it was found that the so called specific soluble substance liberated by the S form and which is held to be responsible for type specificity, was heat stable. When 24 hour cultures of *B. coli-communis* were treated according to the method of Nichols and Smith,²⁵ a highly specific, heat stable filtrate was recovered which was accepted as corresponding to the specific soluble substance reported for various other

organisms. This material gave a precipitation reaction only with S immune serum. Ordinary tests for protein were negative. The substance was toxic and produced death in guinea-pigs. It has not been tested for antigenic properties. No traces of the substance could be demonstrated in R cultures and loss of this property accompanies the $S \rightarrow$ change. The intermediate, O cultures have not been tested for this substance as yet.

SUMMARY

Microbic dissociation has been produced in *B. coli-communis* and the chief dissociants studied. The S and R forms show a distinct and characteristic difference in colony form and cell morphology. The O form represents an intermediate stage and resembles S or R according to the length of time of cultivation on phenol medium. The $S \rightarrow R$ change may be produced by aging, providing the ultimate reaction of the culture medium is alkaline.

An acid medium produced by the utilization of sugar completely inhibits dissociation. Cultures continue to yield S forms until time of death.

Cultivation of the S form in homologous immune serum readily brought about the $S \rightarrow R$ change. The R form produced was not permanent, however, and was soon replaced by the RS colony on alkaline agar plates. The $R \rightarrow S$ change was more difficult to effect than was the $S \rightarrow R$. Reversion was only brought about by cultivation of the R form in homologous R immune serum or by rapid passage in lactose broth.

Only slight differences in cultural reactions of the S and R forms were demonstrated. A distinct difference in the virulence of the two forms was demonstrated. The lethal dose of S was 15 times that of R. Definite protection against a lethal plus dose of S was conferred on guinea-pigs by immunization with R.

The S and R form differed in antigenic properties. The S immune serum contained immune bodies for both S and R while the R immune serum contained agglutinins only for R. A specific soluble substance giving highly specific reactions with S immune serum was demonstrated in the S cultures. No trace of this substance was found in the R cultures.

BIOGRAPHY

The writer was born in Slater, Missouri, where she received her secondary and high school education. She entered the University of Missouri in 1909 and graduated in 1913 with the degree of A. B. She returned to the University of Missouri in 1919 as bacteriologist in the Public Health Laboratory, and in 1922 received the degree of A. M. Her graduate work was done under the direction of Dr. M. P. Ravenel and the thesis is listed in the publications given below.

The year of 1925-26 was spent at the University of Michigan and the year of 1926-27 at the University of Missouri where she completed the requirements for the Ph. D. degree.

The writer has held the following positions: Bacteriologist, Eli Lilly and Co., 1917-19; Bacteriologist, Public Health Laboratory, University of Missouri, 1919-24; Assistant Professor Medical Bacteriology, University of Missouri, 1924-25.

The following list of publications represent the results of her research work to the present date:

(1) Nonspecific Cross Fixation of Complement with Wassermann and Tuberculosis Antigens (Thesis for A. M. degree)—*American Review of Tuberculosis*, 1922, Vol. 6, p. 192.

(2) The Wasserman and Kahn Tests Compared in 900 Cases—*American Journal of Public Health*, 1923, Vol. 13, p. 472.

(3) A Study of Sera of Rabbits Immunized against Globulins from Human Sera—*Journal of Immunology*, 1924, Vol. 9, p. 427.

(4) The Essential Unity of the Wassermann and Kahn Precipitation Tests (with Dr. M. P. Ravenel)—*Southern Medical Journal*, 1925, Vol. 18, p. 491.

(5) A Family of Typhoid Carriers—*American Journal of Public Health*, 1925, Vol. 23, p. 273.

